Investigating the mechanism behind the feedback regulation of the Exon Junction Complex component MAGOH

Undergraduate Research Thesis

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by Abigail Nega

The Ohio State University

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Project Advisor: Dr. Guramrit Singh, Department of Molecular Genetics

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Abstract

RNA binding proteins are critical for regulation of RNA function, and therefore maintaining appropriate levels of these proteins is important for cellular regulation. I focus on MAGOH, an essential subunit of the Exon Junction Complex (EJC) that is necessary for stable binding of the EJC to mRNA. Like all key regulatory proteins, MAGOH levels in the cell are tightly controlled. Reductions in MAGOH can lead to defects in embryonic development. We have found that, in human embryonic kidney (HEK293) cells, when an externally introduced copy of *MAGOH* (exogenous *FLAG-MAGOH*) is forcibly expressed from a tetracycline-inducible promoter, it causes the levels of endogenous MAGOH protein to go down. Thus, there is a feedback mechanism that maintains appropriate levels of MAGOH. The molecular details of this mechanism remain largely unknown and are the subject of my research. Here, I test if the regulation of MAGOH occurs at the transcriptional or post-transcriptional level. My results show that when increasing amounts of *FLAG-MAGOH* mRNA and protein are expressed in the cell, the endogenous *MAGOH* mRNA and pre-mRNA levels are unchanged. This data suggests that neither the degradation nor the synthesis of endogenous *MAGOH* mRNA is affected by more FLAG-MAGOH protein expression. Interestingly, I find that when cells are treated with a proteasome inhibitor (MG-132), the endogenous MAGOH protein does not decrease as observed in the untreated cells. Therefore, MAGOH protein levels are regulated by proteasome-mediated degradation of the protein. Additionally, the incorporation of MAGOH into the EJC seems to play a role in its regulation.

Introduction

Once DNA is transcribed into RNA, there are proteins that bind to RNA which play a crucial role in controlling RNA function. Dysregulation of these RNA-binding proteins can lead to disease, as they are important to normal cell function. The Exon Junction Complex (EJC) is a set of RNA-binding proteins that is deposited upstream of exon-exon junctions during pre-mRNA splicing and plays an important role in post-transcriptional regulation of mRNA. The EJC core has three main proteins: MAGOH, Y14 (also called RBM8A), and EIF4A3. MAGOH forms a heterodimer with the Y14 protein. This heterodimer then binds to the EIF4A3 protein. If MAGOH does not bind to EIF4A3, then it cannot form an EJC. The EJC plays a part in the post-transcriptional regulation of mRNA, including mRNP packaging, export, and localization, as well as nonsense-mediated decay, and translation (Woodward et al., 2016). So, having the proper ratios of the EJC core proteins is important to have normal functioning of the EJC and the cell.

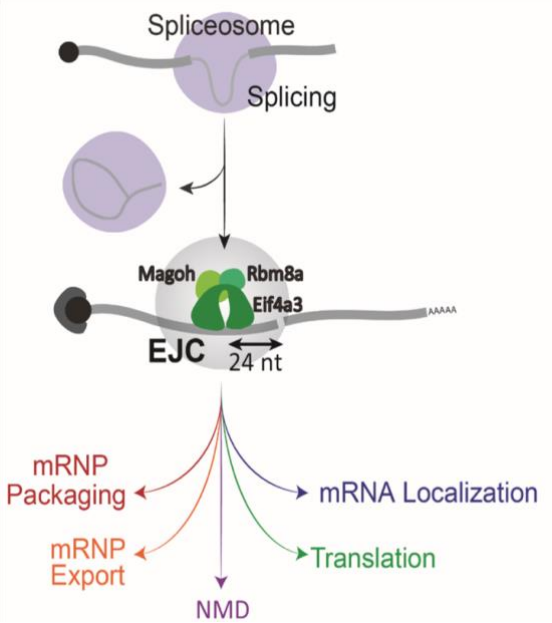


Figure 1. Schematic of EJC proteins and various functions of the EJC (Woodward et al., 2016)

MAGOH is an extremely conserved protein that regulates gene expression in all multicellular eukaryotes, including humans. Given that MAGOH is essential for stable binding of EJC to RNA, reduction in MAGOH levels can lead to defects in embryonic development and other negative consequences for the organism.

There are two *MAGOH* genes in mammals, *MAGOH* and *MAGOHB*. The proteins encoded by these genes are nearly identical and perform the same functions in the cell (Singh et al., 2013). One of these functions is to stimulate the nonsense mediated decay (NMD) pathway, which degrades erroneous mRNAs. Since MAGOH plays an important role in NMD, the levels of MAGOH present are critical to making sure the right transcripts are translated into proteins and ones that need to be removed are degraded. When the levels of either *MAGOH* or *MAGOHB* mRNA are decreased, NMD operated as usual; however, the loss of both proteins resulted in a reduction in NMD (Singh et al., 2013). Thus, it is possible that a regulatory pathway would be important in maintaining the right overall levels of MAGOH between the two genes, which ensures that NMD occurs as needed.

MAGOH also plays a role in brain development. Mice with one copy of a mutant *Magoh* allele and one wild type copy displayed decreased body size and microcephaly (Silver et al., 2010). This indicated that MAGOH haploinsufficiency has extremely negative consequences for the mice. Once again, correct amounts of MAGOH are necessary to an organism's health and proper development.

Like MAGOH, the other proteins involved in the EJC must be present in appropriate levels for normal cell function. In one study, when EIF4A3 was knocked down in *Xenopus laevis*, the embryos experienced paralysis, as well as defects in cardiac and pigmentation development (Haremaki et al., 2010). The same study showed that Y14 and MAGOH

knockdown exhibited similar phenotypes. Like in *Xenopus*, mutant embryos of zebrafish that lack MAGOH or Y14 have pericardial edema, paralysis, and neuron defects (Gangras et al., 2019). Thus, all three core EJC proteins need to be expressed at proper levels, in order to perform their role in the EJC.

To analyze MAGOH levels within a cell, I have been working on comparing endogenous MAGOH expression with exogenous FLAG-MAGOH expression. Our lab had observed in the past that when FLAG-MAGOH is expressed in the cells, the endogenous MAGOH protein levels are reduced, suggesting feedback regulation (Singh et al., 2012). FLAG-MAGOH is a tagged version of MAGOH that can be detected independently of the endogenous MAGOH that naturally occurs in the cell.

Regulation of MAGOH could occur at the transcriptional or post-transcriptional level. It could be regulated at the mRNA level, affecting the synthesis or the degradation of *MAGOH* mRNA. Another option would be regulation at the protein level, meaning the synthesis or degradation of the MAGOH protein is being controlled. Finding the mechanism behind this regulation will be useful for further understanding of control of MAGOH inside cells, and how the necessary stoichiometry of RNA binding proteins is maintained.

There are various mechanisms that are used to regulate stoichiometry. At the transcriptional level, if mRNAs from an exogenous copy of a gene are increased, mRNA synthesis from the endogenous genes could decrease or mRNA transcripts could be degraded more rapidly. In one study that investigated dosage-compensated genes, the dosage compensation was found to have not occurred at the transcriptional level, as mRNA levels of the endogenous genes did not change when gene copy numbers increased (Ishikawa et al., 2017). At the post-transcriptional level, translational control is a possible point of regulation, where

endogenous proteins are not synthesized as frequently in the presence of exogenous protein overexpression. In the case of the POP5 protein, there was no change in translational efficiency (Ishikawa et al., 2017). Another post-translational mechanism that can regulate stoichiometry is the degradation of proteins via the ubiquitin-proteasome system. In this pathway, ubiquitin is linked to at least one lysine in the protein, which makes the protein a target for a proteasome that will degrade it. Cells that were defective for proteasomes had weaker dosage compensation and were found to have greater amounts of ubiquitinated protein (Ishikawa et al., 2017). This suggests the proteasome plays a main role in the regulation of some proteins.

Incorporation of MAGOH into the EJC could be linked to its feedback regulation. In support of this idea, we have also noted in zebrafish that when MAGOH levels are reduced in the cell, Y14 levels drop too; the reverse is also true (Gangras et al, 2019). To test this theory, mutant versions of MAGOH that do not bind to the EJC are required. This can be achieved by using a PCR-based approach where primers will mutate amino acids of *MAGOH* that are important for binding to EIF4A3 (Gehring et al., 2005). Since MAGOH interacts with multiple proteins in the EJC, different mutants can be tested to see which interaction, if any, are important.

Results

I. MAGOH undergoes feedback regulation

To study MAGOH functions in human cells, our lab uses HEK293 Flp-In TRex cells where an additional copy of *FLAG-MAGOH* cDNA is stably integrated into the genome (Figure 2). The expression of this cDNA can be induced by addition of tetracycline (tet).

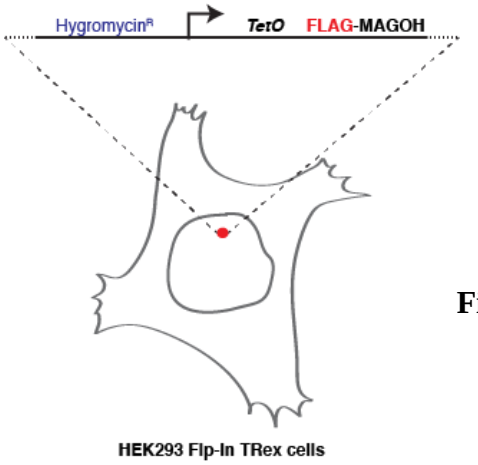
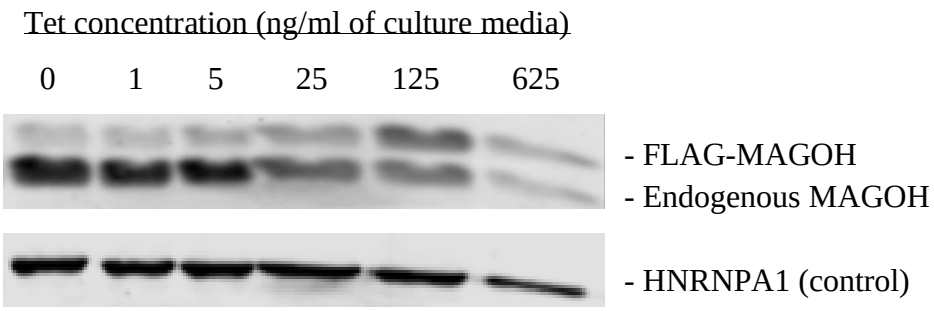


Figure 2. Diagram of FLAG-MAGOH locus. Credit to Dr. Guramrit Singh.

To begin testing the regulation of MAGOH levels, I first confirmed the presence of a feedback mechanism. Increasing amounts of tet were added to HEK293 cells in order to force extra expression of MAGOH from an externally introduced copy of the gene (exogenous *FLAG-MAGOH*). This overexpression of FLAG-MAGOH protein could then be compared to endogenous MAGOH levels using a Western blot, as the FLAG-tagged copy migrated slower than the endogenous copy (Figure 3A). I found that the levels of endogenous MAGOH protein go down, when FLAG-MAGOH levels go up. Higher concentrations of tet made cells express more FLAG-MAGOH protein and less endogenous MAGOH than cells induced with lower concentrations of tet (Figure 3B). Thus, there must be a feedback mechanism that maintains appropriate levels of MAGOH.

A.



B.

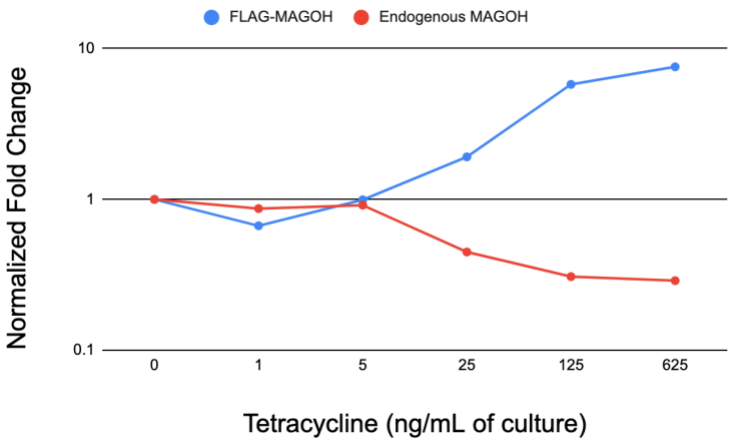


Figure 3.

A. Western blot showing the levels of FLAG-MAGOH protein and endogenous MAGOH protein with increasing tet concentration.

B. Quantitation of FLAG-MAGOH and endogenous MAGOH fold change. MAGOH and FLAG-MAGOH signals were normalized to HNRNPA1, then the fold change was calculated. As FLAG-MAGOH increases, endogenous MAGOH decreases.

This blot is representative of three independent experiments.

II. MAGOH regulation does not occur at the RNA level

Once the existence of a mechanism for MAGOH regulation was supported, finding the timing of the regulation was the next step. One possible point of regulation was at the RNA level, either by decreasing synthesis or increasing degradation of mRNA encoding endogenous *MAGOH*. To test these possibilities, I designed primers that allowed quantification of endogenous *MAGOH* mRNA and *MAGOH* pre-mRNA, using qPCR. *MAGOH Intron 1* is indicative of pre-mRNA levels of *MAGOH* and will represent the effect that increased *FLAG-MAGOH* has on *MAGOH* mRNA synthesis. The qPCR analyses show that if *FLAG-MAGOH* expression is increased *MAGOH* pre-mRNA transcript levels are not significantly changed, suggesting that mRNA synthesis is not affected (Figure 4, Right Bars). Endogenous *MAGOH* mRNA levels also are not changing, so it is unlikely that mRNA degradation is altered (Figure 4, Left Bars). These results suggest that *MAGOH* mRNA is neither being synthesized less nor being more rapidly degraded, implying regulation of MAGOH is not at the RNA level.

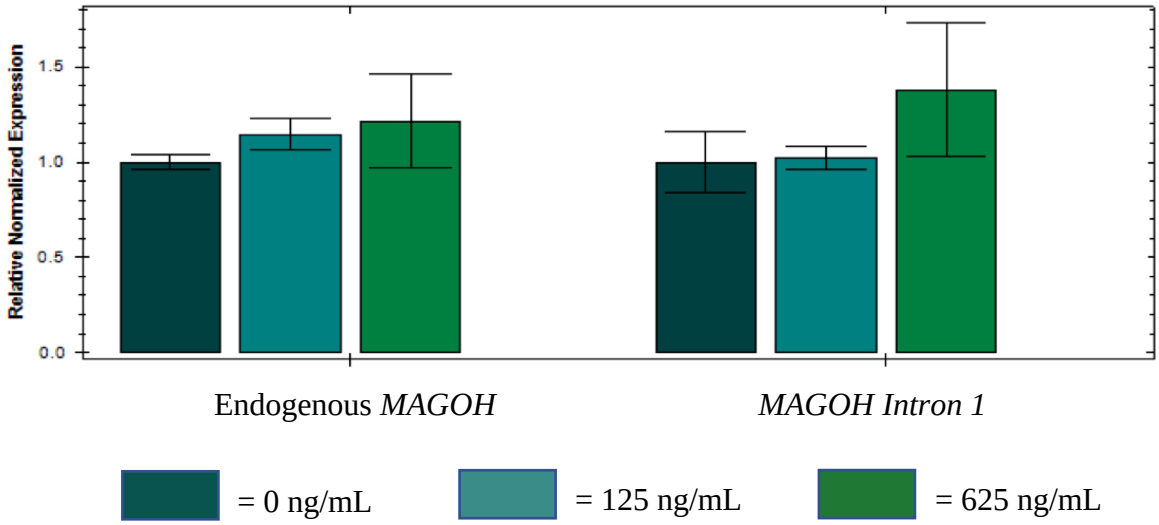


Figure 4. qPCR data showing endogenous *MAGOH* mRNA and *MAGOH Intron 1* levels with various tetracycline concentrations.

Left Bars: *FLAG-MAGOH* overexpression does not significantly change endogenous *MAGOH* mRNA levels.

Right Bars: *MAGOH* pre-mRNA expression, represented by *MAGOH Intron 1*, is not significantly affected when tetracycline concentration increased *FLAG-MAGOH* expression.

The shown data is representative of three independent experiments.

III. FLAG-MAGOH expression suppresses MAGOH at the protein level

I examined if MAGOH could be regulated at the protein level when excess FLAG-MAGOH is produced. To test this possibility, cells treated with tet at different concentrations to produce varying levels of FLAG-MAGOH were also treated with different concentrations of a proteasome inhibitor, MG-132. The proteasome inhibitor stops the proteasome from degrading any proteins, allowing us to see the amount of MAGOH protein present in the cells. Protein levels of endogenous MAGOH and FLAG-MAGOH were analyzed using a Western blot (Figure 5A). When compared to cells untreated with the proteasome inhibitor (Figure 5B), adding the proteasome inhibitor at 10 μM appears to not have an effect (Figure 5C). However, treating cells with the proteasome inhibitor at 25 μM (Figure 5D) lessens the reduction of MAGOH in the cells. To test if MG-132 significantly increased the endogenous MAGOH levels, I compared endogenous MAGOH levels with no inhibitor to endogenous MAGOH levels with 25 μM of inhibitor, using the 125 ng/mL and 625 ng/mL of tet data points (highlighted in red rectangles in Figure 5A) in a paired T-test. I found a statistically significant difference between levels of endogenous MAGOH in the cells without inhibitor and cells with inhibitor when $\alpha = 0.05$, as the p-value was 0.0145. This means that proteasome inhibition interferes with the FLAG-MAGOH induced decrease in endogenous MAGOH levels. Thus, the decrease in endogenous MAGOH in the presence of increased FLAG-MAGOH appears to be due to MAGOH proteins being targeted for degradation.

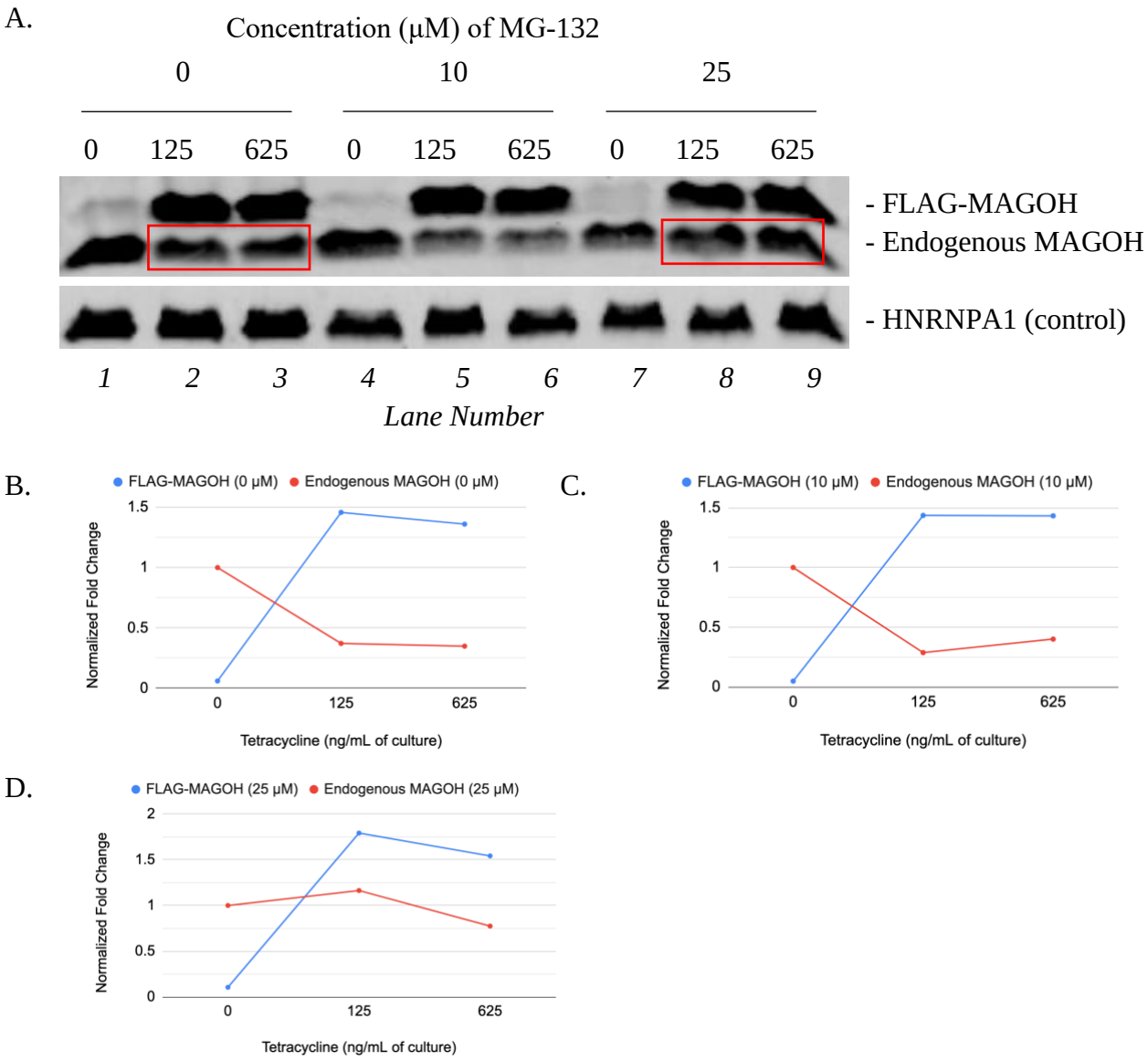


Figure 5. Proteasome Inhibitor and Protein Levels

A. Western blot showing the FLAG-MAGOH and endogenous MAGOH protein levels from MG-132 treated and untreated cells. HNRNPA1 was probed to control for total protein levels.

B. Quantitation of FLAG-MAGOH and endogenous MAGOH protein fold change from untreated cells (lanes 1-3). HNRNPA1 used to normalize MAGOH and FLAG-MAGOH levels.

C. Quantitation as in B, from cells treated with 10 μM MG-132 (lanes 4-6).

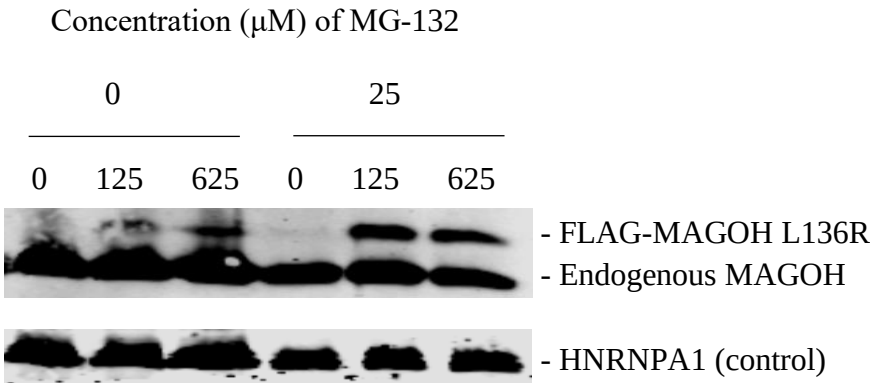
D. Quantitation as in B, from cells treated with 25 μM MG-132 (lanes 7-9).

This blot is representative of two independent experiments.

IV. Incorporation of MAGOH into EJC may affect regulation of its protein levels

Next, I wanted to test if MAGOH's interactions with proteins in the EJC play a role in its regulation. For this purpose, two different *FLAG-MAGOH* mutant cell lines were created. One expressed a mutant, FLAG-MAGOH F134A/K130E, which can bind to Y14 and not to EIF4A3; another expressed a mutant, FLAG-MAGOH L136R, which binds neither to Y14 nor EIF4A3. Both mutants do not assemble into the EJC. When expression of exogenous FLAG-MAGOH L136R was induced, the endogenous MAGOH protein levels were not changed in the absence or presence of MG-132 (Figure 6). In addition, FLAG-MAGOH L136R levels increase in cells treated with MG-132, when compared to untreated cells. This suggests that since they cannot bind to Y14 and are free in the cell, they are being degraded. It remains to be tested if the expression of FLAG-MAGOH F134A/K130E also does not subject endogenous MAGOH to proteasome-dependent degradation. From these results, I conclude that the regulation of MAGOH is either linked to its incorporation into the EJC or the Y14/MAGOH heterodimer.

A.



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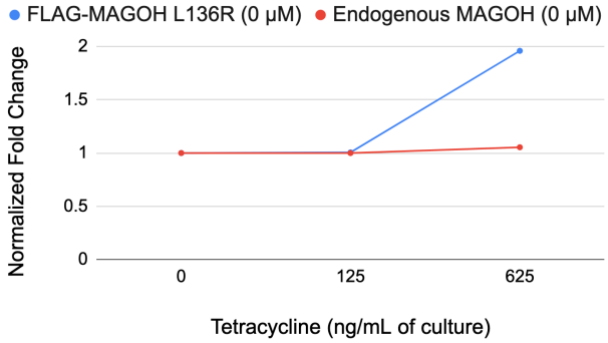

C.

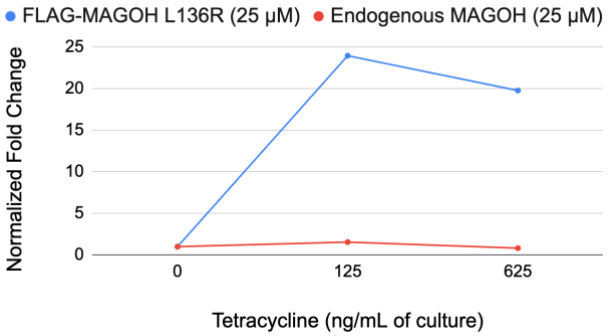

Figure 6. Proteasome Inhibitor and Protein Levels in *FLAG-MAGOH L136R* cells

A. Western blot showing the FLAG-MAGOH, endogenous MAGOH protein and HNRNPA1 levels from MG-132 treated and untreated cells.

B. Quantitation showing normalized fold change of FLAG-MAGOH and endogenous MAGOH protein levels from untreated cells (lanes 1-3).

C. Quantitation as in B, from cells treated with 25 µM of MG-132 (lanes 7-9).

This experiment was only done once.

Discussion

MAGOH protein levels are tightly regulated to preserve the proper functioning of the cell. This regulation does not happen at the RNA level, as *MAGOH* mRNA and pre-mRNA transcript levels are not affected by overexpression of *FLAG-MAGOH*. Instead, it occurs at the protein level, where MAGOH proteins are being degraded after being targeted to the proteasome. Exogenous FLAG-MAGOH can form heterodimers with Y14, putting it in competition with the endogenous MAGOH. Overexpression of FLAG-MAGOH then causes an excess of free MAGOH in the cell. Previous work suggests that MAGOH is more likely to be affected by an overexpression of its exogenous counterpart than Y14 is to be affected by an overexpression of exogenous Y14 (Ma et al., 2019). This suggests that free MAGOH protein is unstable. The decreased levels of endogenous MAGOH protein as FLAG-MAGOH increased found in my experiments supports this conclusion, which is why MAGOH is marked for degradation by the proteasome.

One question that arises from this is how MAGOH is targeted to the proteasome. As proteasome substrates are known to be modified with ubiquitin, it is possible to identify the potential amino acids in the MAGOH protein sequence that will undergo ubiquitination. A search for MAGOH in a catalog of human ubiquitinated proteins (Huang et al., 2016) revealed multiple lysine amino acids, at positions 41, 61, and 114, as possible sites of ubiquitination with a high confidence. It is likely that after one or more of these sites undergo ubiquitination, the proteasome targets MAGOH for degradation. Further research can be done into which position or positions are ubiquitinated and thus most relevant to proteasome-mediated degradation of MAGOH.

Regulation of MAGOH may be tied to its assembly into the MAGOH/Y14 heterodimer or incorporation into the EJC. The results of my experiments show that if overexpressed FLAG-MAGOH cannot bind to Y14, it does not cause the endogenous MAGOH to be downregulated. Supporting my results, another study showed that exogenous MAGOH L136R, when induced into overexpression using doxycycline, does not significantly affect expression of endogenous MAGOH (Ma et al, 2019). One possible explanation is that in the *FLAG-MAGOH L136R* mutant, exogenous MAGOH does not compete with endogenous MAGOH to form the heterodimer, and therefore no regulation of endogenous MAGOH is necessary. This suggests that either being able to form the heterodimer or being part of the EJC is crucial to the regulation of MAGOH protein. More research needs to be done with the *FLAG-MAGOH K130E/F134A* mutant. Comparing the *FLAG-MAGOH K130E/F134A* and *FLAG-MAGOH L136R* mutants will to give insight into whether FLAG-MAGOH not forming the EJC or not forming the heterodimer is more significant to MAGOH regulation.

In conclusion, my research has shown that MAGOH is regulated by a feedback mechanism driven by proteasome-mediated degradation, and its role in the EJC likely influences this regulation. This is significant as it provides insight into the control of MAGOH and into a key mechanism that may maintain appropriate stoichiometry for other proteins in protein complexes, as well.

Materials and Methods

Cell Culture

Human embryonic kidney (HEK293) Flp-In TRex cells were cultured at 37°C in medium composed of Dulbecco's Modified Eagle's Medium (DMEM), with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Cells were treated with tetracycline and/or MG-132 as necessary.

Generation of Mutant Cell Lines

To generate *FLAG-MAGOH K130E/F134A* and *FLAG-MAGOH L136R* cells, primers were first created for *MAGOH K130E*, *MAGOH F134A*, and *MAGOH L136R*. To construct the mutant plasmids, site-directed mutagenesis PCR was done with the mutant primers and *pcDNA5 FLAG-MAGOH* as a template, then digested with DpnI. For the double mutant, the *K130E* plasmid was constructed first, then used as a template with *F134* primer. *E. coli* competent cells were transformed with the mutant plasmids. Colonies were picked for overnight cultures, then DNA was isolated using a standard miniprep protocol. Sanger sequencing was performed to confirm the mutations. HEK293 Flp-In TRex cells were then co-transfected at a ratio of 9:1 pOG44:*FLAG-Magoh* plasmid using TransIT Transfection Protocol. Transfected cells were selected for stable transformants that are hygromycin resistant over many days, replacing the media and adding hygromycin B as necessary, until colonies visible to naked eye were formed. These cells were then trypsinized and grown to confluence under selection.

Protein Analysis

HEK293 Flp-In TRex cells stably transformed to express FLAG-MAGOH proteins were grown in 12-well plates, induced with tetracycline for 16-20 hours to express the FLAG-tagged protein, and were harvested in 1 mL of PBS. 100 μL was reserved in tubes from which to

precipitate for protein extraction. Cells were lysed with 20 μL of 2x SDS loading buffer and 1 μL of 0.1M DTT. The proteins were separated on 15% SDS-PAGE gels and transferred to a membrane, which was then incubated with the appropriate primary antibodies for MAGOH, FLAG-MAGOH, and HNRNPA1 for protein detection. Blots were incubated with infra-red dye conjugated secondary antibodies for visualization and quantitation using LI-COR Odyssey scanner and Image Studio software.

RNA Analysis

HEK293 Flp-In TRex cells induced for FLAG protein expression in 12-well plates were harvested and re-suspended with 1 mL of PBS. 900 μL were reserved in tubes from which to precipitate for RNA extraction. RNA was isolated using a Trizol reagent protocol. cDNA was synthesized using SuperScript III First Strand Synthesis protocol. qPCR was performed in triplicate and the Bio-Rad software was used to assess expression of endogenous *MAGOH* mRNA and *MAGOH* pre-mRNA, and β-actin mRNA was used as a control.

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